# Regulation of ARNO nucleotide exchange by a PH domain electrostatic switch

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**ARNO is a member of a family of guanine nucleotide exchange factors that activate small GTPases called ADP-ribosylation factors (ARFs) [1–3], which regulate vesicular trafficking and, in one case (ARF6), also regulate cortical actin structure [4]. ARNO is located at the plasma membrane, and in the presence of activated protein kinase C (PKC) can induce cortical actin rearrangements reminiscent of those produced by active ARF6 [5–8]. High-affinity binding of ARNO to membranes, which is required for exchange activity, is mediated cooperatively by a pleckstrin homology (PH) domain and an adjacent carboxy-terminal polybasic domain [3,9]. ARNO is phosphorylated *in vivo* by PKC on a single serine residue, S392, located within the carboxy-terminal polybasic domain. Mutation of S392 to alanine does not prevent ARNO-mediated actin rearrangements, suggesting that phosphorylation does not lead to ARNO activation [6]. Here, we report that phosphorylation negatively regulates ARNO exchange activity through a 'PH domain electrostatic switch'. Introduction of a negatively charged phosphate into the polybasic domain reduced interaction of ARNO with membranes both *in vitro* and *in vivo*, and inhibited exchange *in vitro*. This regulated membrane association is similar to the myristoyl electrostatic switch that controls membrane binding of the myristoylated alanine-rich C kinase substrate (MARCKS) [10], but to our knowledge is the first demonstration of an electrostatic switch regulating the membrane interaction of a protein containing a PH domain. This mechanism allows regulation of ARNO lipid binding and exchange activity at two levels, phosphoinositide-dependent recruitment and PKC-dependent displacement from the membrane.**

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Received: 21 June 1999
Revised: 20 August 1999
Accepted: 9 September 1999

Published: 11 October 1999

Current Biology 1999, 9:1173–1176

0960-9822/99/$ – see front matter

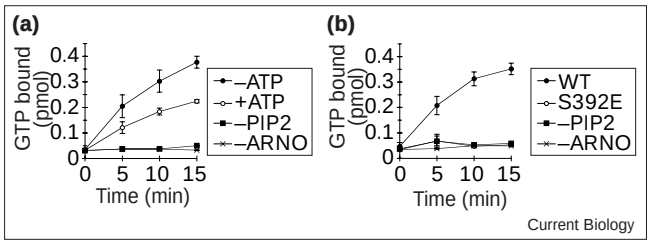

## Results and discussion

We have shown previously that ARNO is phosphorylated within its polybasic domain. Because this domain contributes to membrane lipid binding [9], we hypothesized that phosphorylation might inhibit ARNO activity by displacing it from the membrane. To test this hypothesis, we measured the effect of ARNO phosphorylation on its nucleotide exchange activity *in vitro*. Nucleotide exchange was assayed in the presence of unilamellar phospholipid vesicles containing either a mixture of phosphatidylcholine (PC) and phosphatidylserine (PS), or the same lipids supplemented with phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$). As shown previously [11], ARNO activity was dramatically enhanced in the presence of polyphosphoinositides, as little exchange activity was detected in the absence of this lipid (Figure 1). Exchange occurred in the presence of liposomes containing either $PI(4,5)P_2$ ($EC_{50}$ = 0.4%) or phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$, $EC_{50}$ = 0.025%; data not shown).

The effect of ARNO phosphorylation on nucleotide exchange activity was tested by incubating ARNO with PKC in the presence or absence of ATP. Phosphorylated ARNO exhibited significantly reduced exchange activity compared with non-phosphorylated ARNO (Figure 1a). Nevertheless, ARNO exchange activity was not completely inhibited by phosphorylation, which could be because of incomplete phosphorylation of ARNO by PKC under these conditions. To test this possibility, we replaced the S392 residue with glutamic acid (S392E), thereby producing a protein with a permanent negative charge at this position. The S392E mutant had negligible exchange activity (Figure 1b), suggesting that the residual activity exhibited by phosphorylated ARNO is due to incomplete phosphorylation.

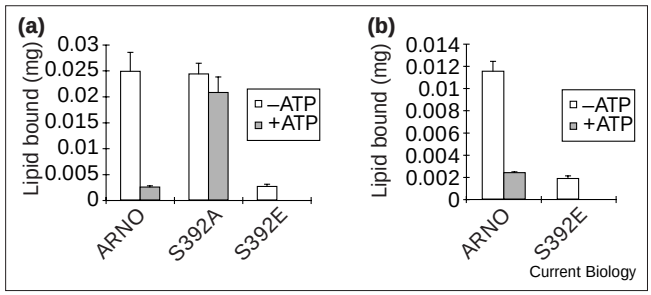
Binding of ARNO to membranes is mediated cooperatively by the PH domain, which binds to polyphosphoinositides, and the polybasic domain, which interacts electrostatically with acidic phospholipids [3,9]. This arrangement of lipid-binding domains is reminiscent of the domain structure of other proteins, such as Src and MARCKS [12,13]. These proteins contain a covalently attached myristoyl group, which inserts into the hydrophobic interior of the membrane bilayer, and a positively

**Figure 1**




Phosphorylation of ARNO by PKC inhibits nucleotide exchange activity towards ARF6. (a) After incubation of ARNO with PKC in the absence or presence of ATP, ARNO exchange activity on ARF6 was assayed as described in the Materials and methods. (b) A negative charge at position 392 inhibits ARNO exchange activity. Wild-type ARNO and the S392E ARNO mutant were tested for exchange activity as described in the Materials and methods. In (a,b), negligible exchange activity was seen in the absence of ARNO or when liposomes lacking $PI(4,5)P_2$ were used in the exchange assay. Data shown are mean ± SEM of triplicate samples.

**Figure 2**




PKC-mediated phosphorylation of ARNO at S392 inhibits binding of ARNO to liposomes. Binding of ARNO to liposomes containing (a) $PI(4,5)P_2$ or (b) $PI(3,4,5)P_3$. ARNO (5 μg), liposomes (0.4 mg/ml) and PKC were incubated in the absence or presence of ATP, and the ARNO–liposome complexes isolated and quantitated as described in the Materials and methods section. Binding of the S392E mutant was tested only in the absence of ATP. Data shown are mean ± SD of triplicate samples and are representative of four experiments.

charged subdomain that interacts electrostatically with acidic phospholipids [12,13]. The basic region of MARCKS contains multiple sites for PKC phosphorylation, and introduction of negatively charged phosphates into the basic domain abrogates interaction of this domain with the membrane [13,14]. Phosphorylation of MARCKS thus leads to translocation of this protein from the membrane to the cytosol [14]. This mechanism of regulated membrane association has been referred to as a myristoyl electrostatic switch [10].

To investigate whether phosphorylation of S392 inhibits ARNO exchange activity by reducing ARNO lipid binding, we examined the binding of ARNO *in vitro* to liposomes containing [$^3$H]PC as a tracer. Binding of ARNO to liposomes was dependent on the presence of phosphoinositides in the liposomes and a functional PH domain in ARNO (data not shown). We tested for the presence of an electrostatic switch by incubating recombinant ARNO and liposomes with PKC in the presence or absence of ATP. As shown in Figure 2a, incubation of wild-type ARNO with PKC and ATP reduced the binding of ARNO to liposomes by 90%. In contrast, incubation of the S392A ARNO mutant with PKC and ATP had little effect on the ability of the recombinant protein to bind liposomes, ruling out any indirect effects of either the kinase or ATP on ARNO–lipid interactions. The S392E mutation also disrupted binding of ARNO to liposomes (Figure 2a). Similar results were obtained when liposomes contained $PI(3,4,5)P_3$ instead of $PI(4,5)P_2$ (Figure 2b). Taken together, these results support the hypothesis that introduction of a negatively charged phosphate group at position 392 is sufficient to destabilize the association of ARNO with the membrane *in vitro*.

To determine whether phosphorylation regulates the association of ARNO with membranes *in vivo*, we examined the distribution of phosphorylated ARNO in metabolically labeled cells. Cells expressing Myc-epitope-tagged wild-type ARNO (Myc–ARNO) were labeled with $^{32}$P-orthophosphate as described in the Materials and methods section and incubated either with or without 500 nM phorbol 12-myristate 13-acetate (PMA) to activate PKC. Immunoblot analysis indicated that Myc–ARNO partitioned into cytosolic and membrane-associated pools (Figure 3a, upper panel), as has been reported for endogenous ARNO [5]. Little phosphorylated ARNO is detected in either fraction in the absence of PMA (Figure 3a, lower panel). Upon PMA treatment, however, phosphorylated ARNO was readily detectable and, importantly, was selectively accumulated in the cytosol. Although a significant fraction of the total ARNO remained associated with membranes in the presence of PMA, this pool was not phosphorylated. As PKC is thought to function only at membrane surfaces, these data suggest that ARNO is phosphorylated while membrane-bound and that phosphorylation induces its translocation to the cytosol.

The effect of a negative charge on lipid binding *in vivo* was confirmed by comparing the localization of ARNO mutants lacking the coiled-coil domain in the presence or absence of the S392E mutation. Membrane association of the S392E mutant was reduced approximately 75% relative to the control, but binding of ARNO to membranes was not completely eliminated by the S392E mutation (Figure 3b). There are several possible explanations. In addition to binding lipid, ARNO may also interact with other membrane-bound proteins. Alternatively, it must be recalled that the S392E mutant retains an intact PH domain. The PH domain of cytohesin-1 in the absence of

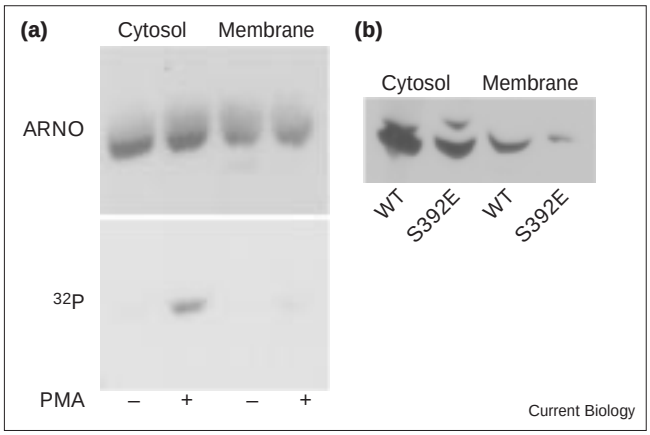
the polybasic domain retains the ability to bind lipid, although with greatly reduced affinity compared with the intact protein [9]. Therefore, although introduction of negative charge into the polybasic domain clearly reduces membrane association, at the levels of expression seen in transiently transfected cells a fraction of the protein might still associate with the membrane.

Several recent studies have indicated that ARNO [15] and its relatives cytohesin-1 [16] and GRP1 [17] can be recruited to the plasma membrane by signals leading to the activation of phosphoinositide (PI) 3-kinase. In each case, the recruitment could be inhibited by the PI 3-kinase inhibitors wortmannin or LY294002, suggesting that D3 phosphoinositides are required for membrane recruitment of these proteins. ARNO can, however, be recruited to actin-rich membrane ruffles by treatment of cells with PMA in a manner that is not inhibited by wortmannin [6]. The most probable explanation is that PKC activation increases availability of $PI(4,5)P_2$-binding sites, either through *de novo* synthesis [18] or by unmasking of pre-existing sites (for example, by displacement of MARCKS) [19]. This recruitment does not require ARNO phosphorylation [6]. It is probable, therefore, that recruitment and phosphorylation are sequential events, phosphorylation occurring only after interaction of ARNO with the membrane.

Regardless of whether ARNO is bound through $PI(4,5)P_2$ or $PI(3,4,5)P_3$ *in vivo*, our data indicate that phosphorylation within the carboxy-terminal polybasic region dramatically reduces the affinity of the protein for membranes containing acidic phospholipids (PS) and either phosphoinositide *in vitro*. Additionally, we have shown that phosphorylated ARNO is exclusively cytosolic *in vivo*, and introduction of a negative charge into the polybasic domain reduces membrane association. Phosphorylation of ARNO by PKC might therefore represent an additional mechanism by which ARNO (and, subsequently, ARF) activation is tightly regulated. In such a model, production or unmasking of polyphosphoinositides would lead to ARNO membrane recruitment and activation of ARF. Although we have suggested that ARF6 is the primary substrate for ARNO because of its plasma-membrane localization, other ARFs would also be potential substrates provided they can localize to the plasma membrane. Phosphorylation of ARNO at the plasma membrane would then terminate the activating signal, resulting in a short burst of ARF activation. As PI 3-kinase activation is not always coupled to PKC activation, it is possible that the duration of the ARF signal might depend on the specific signaling pathway through which ARNO is recruited.

It is also interesting to note that the other members of the ARNO family of GEFs differ in the number of potential phosphorylation sites within the polybasic domain.

**Figure 3**




*In vivo* phosphorylated ARNO is selectively located in the cytosol. (**a**) HeLa cells expressing Myc–ARNO and labeled with $^{32}$P as described in the Materials and methods were incubated in the presence (+) or absence (–) of 500 nM PMA for 20 min. Cells were lysed and separated into cytosol and membranes, and ARNO immunoprecipitated with rabbit anti-ARNO polyclonal antiserum. After separation by SDS–PAGE and transfer to nitrocellulose, radiolabeled ARNO was visualized by autoradiography (lower panel) and western blotting with monoclonal anti-Myc antibody (9E10, upper panel). (**b**) The S392E mutation reduces ARNO membrane association. HeLa cells expressing Myc-tagged wild-type (WT) ARNO or the S392E mutant were fractionated as described in the Materials and methods. ARNO was localized by western blotting with 9E10 antibody. Data shown are representative of multiple experiments.

Although ARNO contains a single serine residue in this region, cytohesin-1 has two serines and a threonine in potential PKC phosphorylation sites, and GRP-1 lacks any potential phosphorylation sites within the basic domain [6]. This suggests that ARNO and cytohesin-1 can be regulated both by production of binding sites at the membrane and by phosphorylation, whereas GRP-1 might be regulated solely by the availability of membrane-binding sites. These exchange factors might thus be uniquely modulated by a variety of signal pathways linking extracellular cues to changes in the actin cytoskeleton, membrane trafficking events or both.

## Materials and methods

### *Materials*

PC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) and PS (1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine) were from Avanti Polar Lipids; $PI(3,4,5)P_3$ (dipalmitoyl-phosphatidylinositol tris-3,4,5-phosphate) from Matreya Inc; $PI(4,5)P_2$ (L-α-phosphatidyl-D-myo-inositol-4,5-bisphosphate) and FuGENE 6 from Boehringer Mannheim; radiolabeled compounds from NEN Life Science; and PKC (calcium-dependent and lipid-dependent α, β and γ isoforms) from Biomol. The rabbit polyclonal antiserum to ARNO has been described previously [5].

### *Liposome production*

Liposomes containing either $PI(4,5)P_2$ or $PI(3,4,5)P_3$ (PC:PS:PIPs 65:30:5 w:w) or no phosphoinositols (PC:PS 70:30) were made by drying lipids out of chloroform under a stream of nitrogen. Dried lipids were resuspended in 20 mM Tris pH 7.5, 120 mM NaCl and

freeze-thawed four times, then extruded through 0.45 μM durapore-PVDF filters (Millipore). Vesicles used in lipid-binding experiments also contained [$^3$H]PC (1.5–2.5 μCi/mg lipid).

### *Recombinant protein production*

$His_6$-tagged ARNO proteins were produced as described previously [6]. Myristoylated ARF6 was produced according to Randazzo *et al.* [20] with the addition of a Sephacryl S-200 gel filtration column as a final step.

### *Guanine nucleotide exchange*

ARNO (100 nM) was incubated with PKC (1.1 ng/μl) in the presence or absence of 200 μM ATP in 20 mM Tris pH 7.5, 60 mM NaCl, 5 mM $MgCl_2$, 250 μM $CaCl_2$, 0.125% BSA, 0.5 mM β-mercaptoethanol, 1 μM PMA and 0.4 mg/ml PI(4,5)$P_2$-containing liposomes at 22°C for 30 min. Exchange was then tested by incubating 10 nM ARNO with 187.5 nM myristoyl-ARF6, 400 nM [$^{35}$S]GTPγS (1.875 Ci/mol) and 0.2 mg/ml liposomes in 20 mM Tris pH 7.5, 1.5 mM $MgCl_2$, 75 μM $CaCl_2$, 100 mM NaCl, 15 μM β-mercaptoethanol at 37°C. At various times, aliquots containing 5.6 pmol ARF6 were removed and exchange stopped by dilution into ice-cold 20 mM Hepes pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl. The amount of [$^{35}$S]GTPγS bound to ARF was determined by a nitrocellulose filter-binding assay.

### *Lipid binding*

ARNO (5 μg, 50 μg/ml), liposomes (0.4 mg/ml) and PKC (0.525 ng/μl) were incubated in the presence or absence of 100 μM ATP in 20 mM Tris pH 7.5, 1.5 mM $MgCl_2$, 75 μM $CaCl_2$, 100 mM NaCl, 15 μM β-mercaptoethanol, and 1 μM PMA for 15 min at 37°C. ARNO and bound liposomes were then isolated by binding to Ni-NTA agarose at 4°C, and washed with cold 20 mM Tris pH 7.5, 120 mM NaCl. The amount of lipid bound to ARNO was quantitated by liquid scintillation counting.

### *Subcellular distribution of phosphorylated ARNO*

HeLa cells were transiently transfected with plasmid expressing Myc–ARNO using FuGENE 6. After overnight incubation in low-serum media (0.5% FBS), cells were phosphate-starved for 2 h in phosphate-free MEM, and labeled for 3 h with $^{32}$P-orthophosphate (0.5 mCi/ml) followed by treatment 500 nM PMA. Cells were lysed and fractionated according to Fleming *et al.* [21]. ARNO was immunoprecipitated with polyclonal rabbit anti-ARNO antiserum and analyzed for the presence of $^{32}$P-ARNO as previously described [6].

### *Subcellular distribution of S392E ARNO*

Plasmids expressing Myc–ARNO lacking the coiled-coil domain either with or without the S392E mutation were transfected into HeLa cells using FuGENE6. Cytosolic and membrane fractions from cells expressing equivalent amounts of ARNO were isolated as described by Nagel *et al.* [9]. Total protein was isolated [22] and analyzed by western blot with monoclonal anti-Myc antibody 9E10.

## Acknowledgements

This work was supported by National Institutes of Health grants AI-32991 to J.E.C. and F32-DK-09924 to L.C.S. and a gift from the Good Samaritan Foundation to J.E.C. S.R.F was supported in part by a predoctoral training grant to the Harvard Medical School Program in Immunology. We thank Alison Criss and Jeff Settleman for careful reading of the manuscript.

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